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Nitric oxide is involved in phosphorus deficiency-induced cluster-root development and citrate exudation in white lupin

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Summary

• White lupin (*Lupinus albus*) forms specialized cluster roots characterized by exudation of organic anions under phosphorus (P) deficiency. Here, the role of nitric oxide (NO) in P deficiency-induced cluster-root formation and citrate exudation was evaluated.

• White lupin plants were treated with the NO donor sodium nitroprusside (SNP) and scavenger or inhibitor of NO synthase under conditions of P deficiency (0 μM) or P sufficiency (50 μM).

• Phosphorus deficiency enhanced NO production in primary and lateral root tips, with a greater increase in cluster roots than in noncluster roots. NO concentrations decreased with cluster root development from the pre-emergent stage, through the juvenile stage, to the mature stage. The P deficiency-induced increase in NO production was inhibited by antagonists of NO synthase and xanthine oxidoreductase, suggesting the involvement of these enzymes in NO production. SNP markedly increased the number of cluster roots. Citrate exudation from different root segments in P-deficient roots was positively correlated with endogenous root NO concentrations.

• These findings demonstrate differential patterns of NO production in white lupin, depending on root zone, developmental stage and P nutritional status. NO appears to play a regulatory role in the formation of cluster roots and citrate exudation in white lupin under conditions of P deficiency.

Introduction

Nitric oxide (NO) is emerging as an important messenger molecule involved in many important physiological processes in plants (Lamattina *et al.*, 2003; Neill *et al.*, 2003; Crawford & Guo, 2005; Besson-Bard *et al.*, 2008; Wilson *et al.*, 2008), ranging from seed germination (Bethke *et al.*, 2006) to stomatal movement (Bright *et al.*, 2006). There is increasing evidence indicating that NO plays a critical role in responses to abiotic stresses in plants, including salinity (Zhao *et al.*, 2007a), drought (Mata & Lamattina, 2001) and freezing (Zhao *et al.*, 2009). Recent studies also revealed that NO plays an important role in responses of plants to essential mineral nutrient conditions such as iron deficiency (Graziano & Lamattina, 2007) and high external nitrate concentrations (Zhao *et al.*, 2007a,b).

NO production in plants can be ascribed to several pathways, and the contribution of each pathway to overall NO production may vary, depending upon plant species, growth stage and environmental conditions (Neill *et al.*, 2003). It has been suggested that nitric oxide synthase (NOS) and nitrate reductase (NR) are two key enzymes catalyzing arginine- and nitrite-dependent NO production in plants, respectively (Neill *et al.*, 2003). NOS-mediated NO

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production in mammalian cells has been well characterized at both cellular and molecular levels (Furchgott, 1995). There are several lines of indirect evidence to support the existence of an NOS-like pathway in plants (Guo *et al.*, 2003; Moreau *et al.*, 2008). However, the plant NOS-like protein remains to be characterized at molecular level (Crawford *et al.*, 2006; Zemojtel *et al.*, 2006; Gas *et al.*, 2009). In contrast to animal cells, there has been ample experimental evidence indicating that NR-mediated NO production plays a role in numerous physiological processes in plants (Desikan *et al.*, 2002; Molodo *et al.*, 2005; Bright *et al.*, 2006). In addition to NOS- and NR-dependent NO production, xanthine oxidoreductase (XOR) is also involved in NO production in mammalian cells, and exists in two interconvertible forms, xanthine dehydrogenase (XDH) and xanthine oxidase (XO) (Harrison, 2002; Martin *et al.*, 2004). However, there is limited information on the role of XOR-dependent NO production in physiological processes in plants. In addition to the enzymatic pathways mentioned above, NO can also be nonenzymatically generated by plants under certain conditions (Bethke *et al.*, 2004).

Phosphorus (P) is an essential element required for plant growth. P deficiency as a result of fixation by soils occurs widely and is one of the limiting factors for plant growth. Plants have evolved numerous adaptive mechanisms to cope with P deficiency. These include modification of root architecture and exudation of organic acids (Ryan *et al.*, 2001; Vance *et al.*, 2003). In this context, white lupin is an ideal material as it forms specialized lateral roots, referred to as 'cluster roots', under P-deficient conditions and these cluster roots are characterized by their capacity to exude huge amounts of organic anions (Neumann & Martinoia, 2002; Vance *et al.*, 2003; Shane & Lambers, 2005). Although NO has been widely recognized to play a role in root development, particularly lateral root development (Gouvêa *et al.*, 1997; Pagnussat *et al.*, 2003; Correa-Aragunde *et al.*, 2004, 2006), little information is available about the interactions between P deficiency and NO in terms of cluster-root formation and citrate exudation in white lupin. In the present study, the effects of P deficiency on NO production in cluster roots at different developmental stages in white lupin were characterized. The possible pathways involved in NO synthesis in P-deficient roots were examined, and the role of NO in citrate exudation from cluster roots was evaluated.

Materials and Methods

Plant growth

White lupin (*Lupinus albus* L. cv Kiev Mutant) seeds were germinated and grown in plastic pots containing 6 l of an aerated nutrient solution (five plants per pot). The solution was composed of (μM): $Ca(NO_3)_2$ (2000), K_2SO_4 (700), $MgSO_4$ (500), KCl (100), H_3BO_3 (10), $ZnSO_4$ (0.5), $MnSO_4$ (0.5), $CuSO_4$ (0.2), $(NH_4)_6Mo_7O_{24}$ (0.01) and Fe-EDTA (20). Phosphorus was supplied at 0 (P_0, deficient) or 50 μM (P_{50}, sufficient) as KH_2PO_4. The pH of the solution was adjusted daily to 5.6, and the solution was renewed every 3 d. Plants were grown in a controlled environment with a light:dark regime of 14 : 10 h, a temperature of 28 : 18°C and a light intensity of 230 μmol m^{-2} s^{-1}.

Measurement of endogenous NO concentrations in roots by confocal microscopy

White lupin plants were pre-cultured in nutrient solution containing either 0 or 50 μM P for varying periods, and then the primary and lateral root tips were sampled at 3, 7 and 14 d after P treatment (DAT). The juvenile, mature and senescent cluster roots shown in Fig. 1 were excised. The endogenous NO concentration was determined using the NO fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2DA) as described by Correa-Aragunde *et al.* (2004). Briefly, the excised root segments were incubated with 20 μM DAF-2DA in 20 mM Hepes-NaOH buffer (pH 7.5) for 40 min. Thereafter the root segments were washed

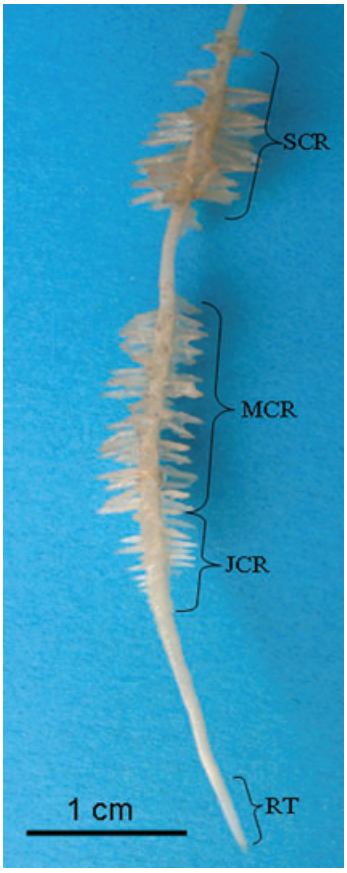

Fig. 1 Sampling of cluster roots at different developmental stages in white lupin (*Lupinus albus*). SCR, senescent cluster roots; MCR, mature cluster roots; JCR, juvenile or young cluster roots; RT, root tip.

thoroughly with the Hepes-NaOH buffer, and DAF-dependent fluorescence from the root segments was visualized using a laser confocal scanning microscope (Te2000-E; Nikon, Tokyo, Japan). The intracellular fluorescence was excited with a 488-nm argon-krypton laser and emission signals at 515 nm were collected. The intensity of fluorescence was quantified using the Te2000-E Nikon software.

For determination of the effects of the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) and antagonists of nitrate reductase (tungstate), NOS (NG-nitro-L-arginine, L-NNA) and XOR (allopurinol) on NO production in roots, root segments from P-deficient and P-sufficient plants were incubated in the solution containing 10 mM c-PTIO for 2 h, and 100 μM tungstate, 0.5 mM allopurinol and 10 mM L-NNA for 12 h, respectively. The endogenous NO production of treated roots was measured as described in the previous paragraph.

Detecting endogenous NO in roots by electron spin resonance (ESR)

White lupin plants were pre-cultured at either 0 or 50 μM P for different periods. The normal and cluster roots of white lupin grown in 0 or 50 μM P were then sampled. Furthermore, pre-emergent (no emergence cluster roots (NECRs)), juvenile and mature cluster roots were excised to determine the changes in endogenous NO content in these roots according to the method described by Cao *et al.* (2005). Briefly, the roots were ground in 0.1 M phosphate buffer (pH 7.4, containing 0.32 M sucrose, 10 mM Hepes, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 5 mM thioaethylenglycol) at 4°C in the presence of quartz sand. The sample was centrifuged at 500 ***g*** for 20 min, and the supernatant was incubated in the solution containing 6 mM $FeSO_4$, 12 mM DETC (*N*,*N*-Diethyldithiocarbamate), 10 mM L-arginine, 10 mM $Na_2S_2O_3$ and 10 mM $CaCl_2$ for 2 h. The reaction was stopped by adding ethyl acetate. The homogenate was centrifuged at 500 ***g*** for 10 min, and the supernatant was used for the detection of the NO signal. The NO signal was measured at room temperature using a Bruker ER200D-SRC spectrometer (Bruker Analytik GmbH, Rheinstetten/Karlsruhe, Germany). The conditions for ESR were as follows: X-band; 100 kHz modulation with 3.2 G amplitude; microwave power 20 mW; central magnetic field 3385 G with scan 400 G; scan time 200 s. NO detected by ESR was expressed as the relative intensity of the signal. The mean height of the three peaks in each signal was taken as the relative intensity of the NO signal (Cao *et al.*, 2005).

Expression of xanthine oxidoreductase (XOR)

Xanthine oxidoreductase (XOR; otherwise referred to as xanthine dehydrogenase (XDH) or xanthine oxidase (XO), which are alternative forms of the same gene product) is also an enzyme recently shown to produce NO (Ichimori *et al.*, 1999; Harrison, 2002). XOR (XDH/XO) catalyzes the reduction of nitrite to NO (Ichimori *et al.*, 1999; Neill *et al.*, 2003). To investigate the contribution of the XOR pathway to NO production, gene expression of *XDH* in cluster roots at different developmental stages under P-deficient or P-sufficient conditions was examined by RT-PCR and RNA blot.

RT-PCR Total RNA was isolated using the RNeasy plant mini kit (Qiagen, Valencia, CA, USA). The cDNA was synthesized from total RNA with the Superscript RT II enzyme (Invitrogen, Carlsbad, CA, USA) and oligo $(dT)_{17}$ primer. The two primer pairs used in the parallel PCR reactions for the *XDH* gene (*LaXDH*) were LaXDH-F 5′-GGGGCAAG GTCTGCATACGAAAG-3′ and LaXDH-R 5′-TCTGT CCGACATCAATAGCTGGG-3′. This primer pair amplifies 470 bp near the 3′ coding region of *LaXDH*. PCR was performed for 26 cycles (94°C for 30 s, 58°C for 30 s and 72°C for 30 s). The primer pair used as a control was tublin-F 5′-GCCTGATGGACAAATGCC-3′ and tublin-R 5′-GCCTGCAAATGTCATAGATAGC-3′. PCR was performed for 26 cycles (94°C for 30 s, 56°C for 30 s and 72°C for 30 s).

RNA gel blot analysis Total RNA was isolated from lupin cluster roots at different stages and from leaf tissue, and then blotted as described previously (Liu *et al.*, 2001). RNA gel blots were performed in duplicate. Membranes were hybridized and washed at high stringency. Filters were exposed to X-ray film (Liu *et al.*, 2001). The lupin ubiquitin gene was used as a quantitative control.

Effect of NO on the number of lateral roots and cluster-root formation

To investigate the effect of NO on cluster-root formation, white lupin plants were cultured in 0 μM P nutrient solution with or without 50 μM SNP for 3 wk. SNP is the most commonly used NO donor in plants, despite some unwanted effects of SNP by-products (Neill *et al.*, 2003), and SNP is used as the exclusive NO donor in numerous studies of NO function in plants (Pagnussat *et al.*, 2003; Creus *et al.*, 2005; Velikova *et al.*, 2005). After treatment with 50 μM SNP, the plants were harvested and the number of lateral roots and cluster roots was determined.

Effect of NO on citrate exudation

To study the effect of NO on citrate exudation from cluster roots, the P-deficient and P-sufficient plants were incubated in 50 μM SNP, 200 μM L-NNA or 100 μM tungstate solutions for 24 h or in 100 μM c-PTIO solution for 2 h.

After the incubation, different root segments (including 0–10 mm at the root tip, juvenile cluster roots, and mature cluster roots) were excised and citrate exudation was collected, according to the method described by Wang *et al.* (2007). The excised root segments were washed thoroughly with deionized water, and root exudates were collected after incubation of the excised roots in an Eppendorf tube containing collection solution for 2 h. After the collection of root exudates, the samples were stored at −20°C for analysis of organic anions. Organic anions in root exudates were analyzed using a reverse-phase HPLC system according to a previous study (Wang *et al.*, 2006). Separation was conducted on a 250 × 4.6 mm reverse-phase column (Alltima C18, 5 Micron; Alltech Associates, Inc., Deerfield, IL, USA). The mobile phase was 25 mM KH_2PO_4 (pH 2.5) with a flow rate of 1 ml min^{-1} at 28°C, and detection of organic anions was carried out at 214 nm.

Results

Effect of P supply on NO production in primary and lateral roots of white lupin

To investigate the effect of P deficiency on NO production in primary roots and lateral roots, the root segments of primary roots and lateral roots with root tips at different growth stages were used to examine the changes in endogenous NO concentrations in lupin roots grown at 0 or 50 μM P (Fig. 2). The fluorescence intensity dependent on the NO-specific fluorescence probe DAF-2DA was greater in P-deficient primary roots than in P-sufficient roots at both 7 and 14 DAT, suggesting that P deficiency induces an increase in the endogenous NO concentration in primary roots. A similar increase in the endogenous NO concentration was observed in the P-deficient lateral roots (Fig. 2). By contrast, NO fluorescent intensity appeared to be relatively weak in primary roots and lateral roots at 3 DAT, indicating less NO production in the early stages of the P-deficient treatment. Interestingly, the primary roots showed a higher DAF-2DA fluorescence intensity than the lateral roots at 3 DAT irrespective of P supply (Fig. 2). There were no significant differences in NO concentrations between primary and lateral roots grown in the P-deficient or P-sufficient solutions at 14 DAT (Fig. 2). Furthermore, no differences in NO concentrations in primary and lateral roots grown in the P-deficient solutions at 7 DAT were observed (Fig. 2).

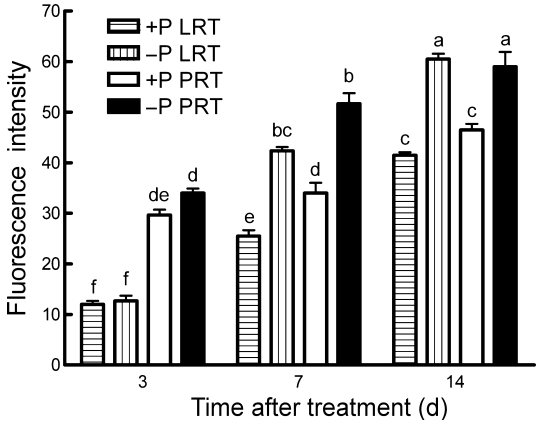

Fig. 2 The signal intensity of nitric oxide (NO) fluorescence in primary root tips (PRT) or lateral root tips (LRT) of white lupin (*Lupinus albus*) receiving 0 μM phosphorus (P) (−P) or 50 μM P (+P) at 3, 7 and 14 d after P treatment (DAT). The normal lateral root tips were sampled at 3 and 7 d when no visible cluster-root formation was found, and the root tips of the lateral root on which very young cluster roots started to emerge were sampled at 14 DAT. The root segments were incubated in 20 μM 4,5-diaminofluorescence (DAF-2DA) in a solution containing 20 mM Hepes-NaOH, pH 7.5, for 40 min, and fluorescence was detected by confocal laser scanning microscopy and quantified using the Te2000-E Nikon software. Data are the mean ± SE of four replicates. Means with different letters are significantly different ($P < 0.05$) with regard to treatment.

NO production during formation of P deficiency-induced cluster roots

To examine the effect of P deficiency on NO production during cluster-root development, cluster roots at different developmental stages were used to investigate the interaction between cluster-root development and endogenous NO concentrations in cluster roots (Fig. 3). The endogenous NO concentration was higher in the juvenile and mature cluster roots than in the senescent cluster roots, as indicated by the greater DAF-2DA fluorescence intensity for the juvenile and mature cluster roots (Fig. 3). In addition, NO concentration and distribution differed during cluster-root development from emergence to the mature stage. For juvenile cluster roots, the newly emerged cluster rootlets had the highest NO concentrations before root hair emergence (Fig. 3a,b). With the development of cluster roots, the base of the rootlets was covered by root hairs and the root tips of the rootlets kept growing. Relatively high NO concentrations were found in the root hairs and growing root tips uncovered by root hairs (Fig. 3c–e). When the rootlets became mature, a higher NO concentration was found in the root hair zone (Fig. 3f). The DAF-2DA-dependent fluorescence was evidently reduced in response to the NO scavenger c-PTIO (Fig. 4a), indicating that DAF-2A-dependent fluorescence intensity is strongly associated with the endogenous NO concentration.

The mammalian NOS inhibitor L-NNA significantly reduced the NO concentration in juvenile and mature cluster roots (Fig. 4). Similar to L-NNA, the XOR inhibitor allopurinol also markedly reduced NO production in P-deficient roots (Fig. 4a,b). However, the nitrate reductase inhibitor tungstate had no effect on NO concentrations in cluster roots (Fig. 4). Given the uncertainties of plant NOS, these results suggest that the NOS-like protein and

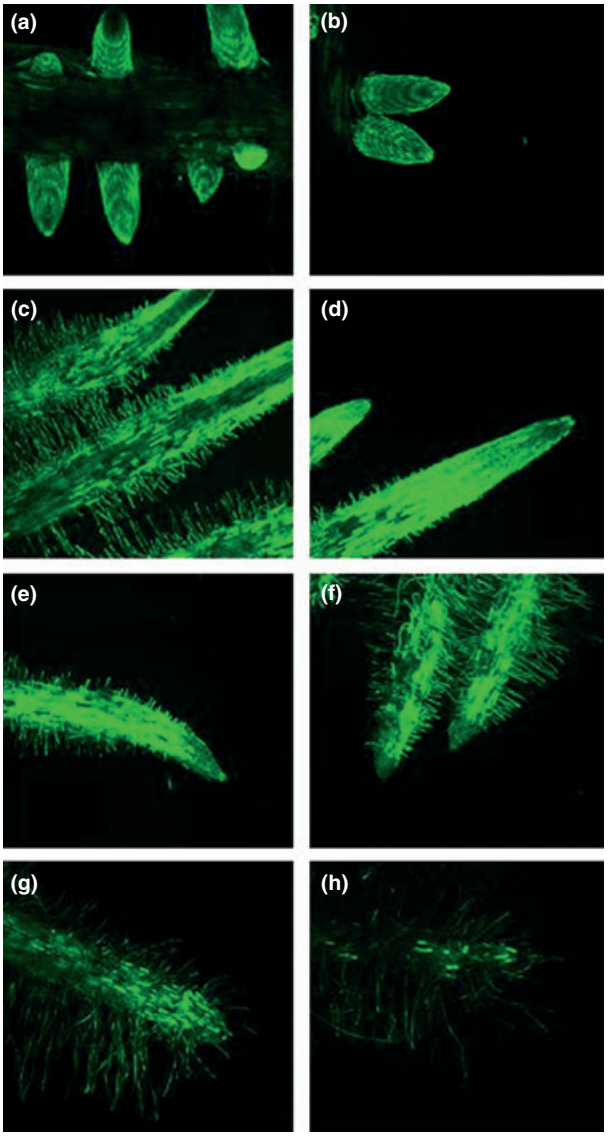

Fig. 3 Nitric oxide (NO) fluorescence of cluster roots of white lupin (*Lupinus albus*) induced by phosphorus (P) deficiency at different growth stages. (a, b) Juvenile cluster roots, (c–e) growing cluster roots, (f) mature cluster roots with root hairs, and (g, h) senescent cluster roots. Cluster root segments were incubated in 20 μM 4,5-diaminofluorescence (DAF-2DA) in a solution containing 20 mM Hepes-NaOH, pH 7.5, for 40 min. Fluorescence was detected by confocal laser scanning microscopy. Magnification: ×10.

XOR may be involved in NO production in P deficiency-induced cluster-root formation, especially at the juvenile and mature stages.

Effect of P deficiency on NO production in cluster roots

In addition to directly labeling the endogenous NO in white lupin roots with a fluorescent probe, the endogenous NO in cluster roots at different growth stages was also detected using the ESR method (Fig. 5). Similar to the confocal imaging results, the NO content in cluster roots was higher than that in the normal roots grown in P-deficient and P-sufficient solutions (Fig. 5a). Further, the P-deficient cluster roots showed greater NO production than the P-sufficient cluster roots, and a similar trend was observed in normal roots (Fig. 5a). Interestingly, there were significant differences in NO concentrations among cluster roots at different developmental stages (Fig. 5b,c). The NO concentration decreased as the cluster roots developed from the pre-emergent to mature stage, with the highest concentration in pre-emergent cluster roots, indicating a higher NO concentration in the early developmental stages of cluster roots (Fig. 5b,c).

Effect of NO on root growth

To determine whether P deficiency-induced root growth is related to the endogenous NO concentration, the endogenous NO concentrations in white lupin roots were experimentally manipulated by treating the roots with the NO donor SNP. A significant increase in the number of the first-order lateral roots was observed upon exposure to SNP for 3 wk (Fig. 6a). In addition to the first-order lateral roots, SNP markedly increased the number of cluster roots (Fig. 6b). However, the stimulating effects of NO on lateral root and cluster root growth were not observed under the conditions of P sufficiency (data not shown), suggesting an interaction between NO and P status in lateral root and cluster root growth. These results indicate that increased endogenous NO is likely to be involved in lateral root growth and cluster-root formation in P-deficient white lupin.

Expression of XOR in cluster roots at different developmental stages under P-deficient or P-sufficient conditions

To further evaluate the contribution of the XOR pathway to NO production, gene expression of white lupin *LaXDH* during cluster root development under P-deficient and P-sufficient conditions was examined by RT-PCR and RNA blot (Fig. 7). *LaXDH* was expressed at all stages of cluster root formation under P deficiency. High expression of *LaXDH* was found in mature cluster roots at zone 5 or in the whole cluster roots grown in P-deficient solutions (Fig. 7d). By contrast, expression of *LaXDH* was strongly repressed in the leaves and root tips. Compared with the P-deficient roots, much less expression of *LaXDH* was observed in cluster roots at all stages under P-sufficient conditions, and expression of *LaXDH* did not differ among cluster roots at different developmental stages (Fig. 7d). The expression of *LaXDH* was strongly suppressed in leaves. This expression pattern was further confirmed by RNA blot (Fig. 7d).

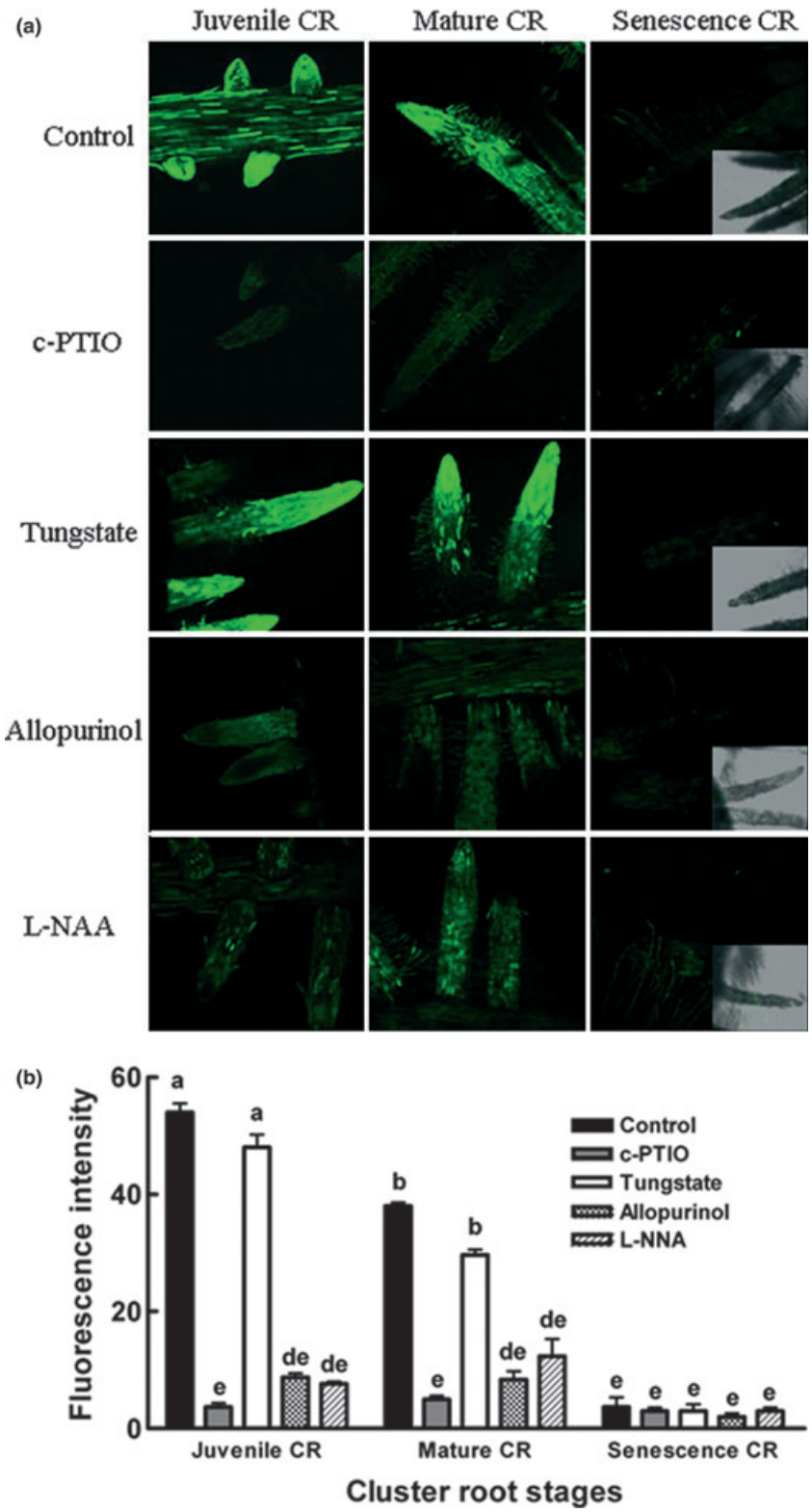

Fig. 4 Effect of the nitric oxide (NO) scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline -1-oxyl-3-oxide (c-PTIO), the nitrate reductase inhibitor tungstate, the xanthine oxidoreductase inhibitor allopurinol and the nitric oxide synthase inhibitor N-nitro-L-arginine (L-NNA) on the fluorescence intensity of NO in cluster roots (CR) of white lupin (*Lupinus albus*) at different developmental stages. (a) The fluorescence intensity of NO is shown in juvenile, mature and senescent cluster roots subjected to different treatments. Cluster root segments were pretreated in 10 mM c-PTIO for 2 h, and 100 μM tungstate, 0.5 mM allopurinol or 10 mM L-NNA solution for 12 h. After the pretreatment, cluster root segments were incubated with 20 μM 4,5-diaminofluorescence (DAF-2DA) in a solution containing 20 mM Hepes-NaOH, pH 7.5, for 40 min, and fluorescence was detected by confocal laser scanning microscopy and quantified using the Te2000-E Nikon software. Insets: black and white pictures show the outline of the specific fluorescence pictures in the column of senescent CR. Magnification: ×10. (b) The fluorescence intensity was quantified in cluster roots at different developmental stages. Data are the mean ± SE of six replicates. Means with different letters are significantly different ($P < 0.05$) with regard to treatment.

Effect of NO on exudation of organic anions

To evaluate the effect of NO on P deficiency-induced citrate exudation from cluster roots, SNP was supplied to different root segments. As shown in Fig. 8(a–c), SNP significantly stimulated citrate exudation from the root tips (0–10 mm), and the juvenile and mature cluster roots. By contrast, the NO scavenger c-PTIO markedly inhibited citrate exudation

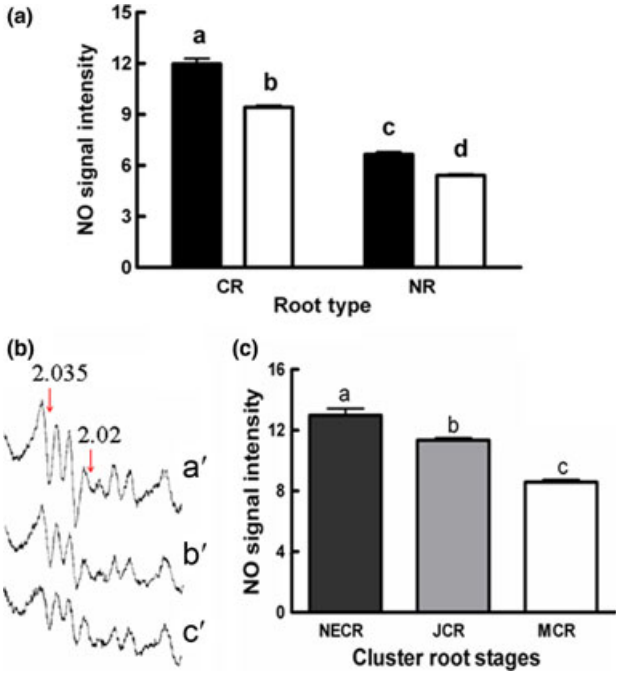

Fig. 5 Nitric oxide (NO) signal intensity of the electron spin resonance (ESR) spectra of the $(DETC)_2$-Fe^{2+}-NO complex in the roots of white lupin (*Lupinus albus*) grown in nutrient solution containing 0 μM phosphorus (P) (−P) or 50 μM P (+P). (a) The NO signal intensity in the normal roots (NR) and cluster roots (CR) of white lupin receiving 0 μM P (−P, closed bars) or 50 μM P (+P, open bars). (b) The ESR spectra of the $(DETC)_2$-Fe^{2+}-NO complex generated in the pre-emergent cluster roots (a′), juvenile cluster roots (b′) and mature cluster roots (c′). The three peaks at g = 2.035 with aN = 12.5 G came from the hyperfine of the NO complex, and the peak at g = 2.02 came from the Cu^{2+} complex. (c) The NO signal in the cluster roots at different developmental stages is shown in the pre-emergent cluster roots (or no emergence cluster roots (NECR)), juvenile cluster roots (JCR), and mature cluster roots (MCR), respectively. Values represent the mean ± SE of three replicates. Means with different letters are significantly different (P < 0.05).

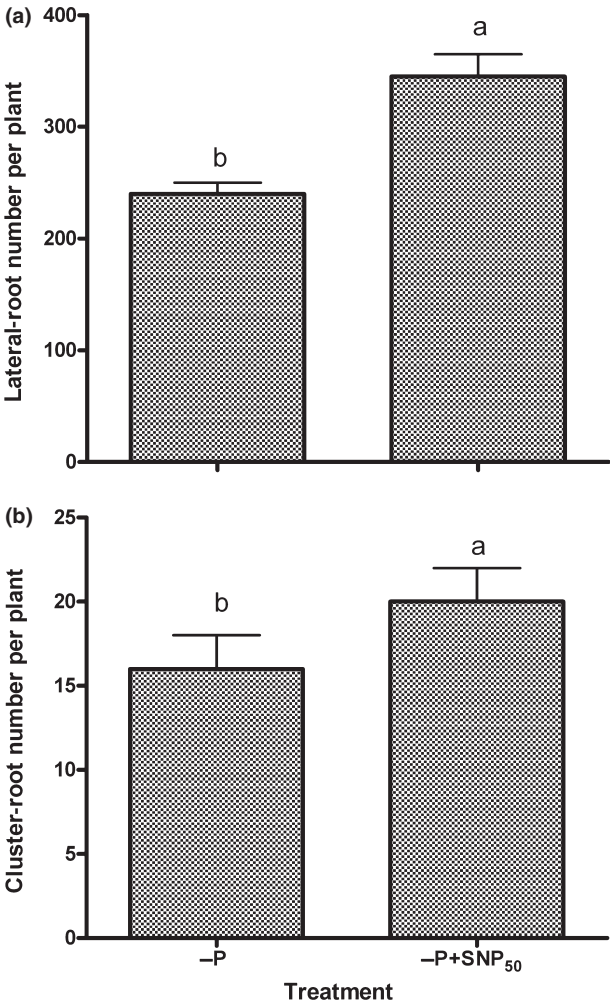

Fig. 6 Effect of the nitric oxide (NO) donor sodium nitroprusside (SNP) on the number of first-order lateral roots (a) and cluster roots (b) in phosphorus (P)-deficient white lupin (*Lupinus albus*). White lupin plants were cultured in 0 μM P (−P) nutrient solution with or without 50 μM sodium nitroprusside (SNP) for 3 wk. After the treatments, the plants were harvested and the number of the lateral roots and cluster roots counted. Data are the mean ± SE of four replicates. Means with different letters are significantly different (P < 0.05) with regard to treatment.

from root tips and juvenile and mature cluster roots by 60, 50 and 60%, respectively (Fig. 8d–f). Moreover, L-NNA has no effect on the citrate exudation of root tips (0–10 mm) and juvenile cluster roots, but it significantly decreased citrate exudation from mature cluster roots (Fig. 9a–c). Compared with the control, the NR inhibitor tungstate strongly stimulated citrate exudation from root tips and juvenile cluster roots, but it did not affect citrate exudation from mature cluster roots (Fig. 9a–c).

Discussion

White lupin is highly adapted to P-deficient soils, and has been widely used to characterize P deficiency-induced cluster-root formation and citrate exudation (Neumann & Martinoia, 2002; Vance *et al.*, 2003; Shen *et al.*, 2004; Shane & Lambers, 2005; Wang *et al.*, 2007). Cluster roots can be divided into juvenile, mature and senescent cluster roots based on their developmental stage (Neumann *et al.*, 1999). There is no substantial difference in anatomical structure between rootlets of juvenile cluster roots and normal lateral roots. However, mature and senescent cluster roots are highly differentiated root structures with very limited longevity, covering by root hairs and lacking active meristems (Watt & Evans, 1999a,b; Neumann & Martinoia, 2002). In the present study, it was found that P deficiency significantly increased the endogenous NO concentration both in the normal lateral roots and in cluster roots as well as in the root hairs of cluster roots (Figs 2, 3, 5). Moreover, P deficiency altered the NO distribution in

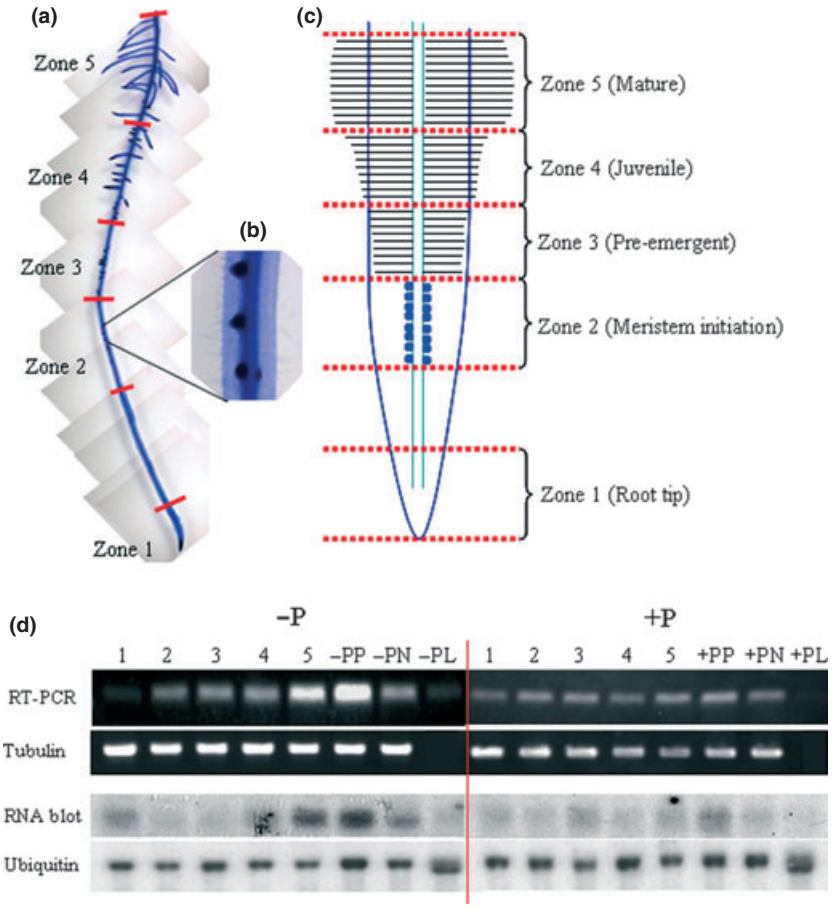

Fig. 7 Sampling root segments and expression of the xanthine dehydrogenase gene (*LaXDH*) in cluster roots of white lupin (*Lupinus albus*) at different developmental stages grown at 0 (–P) or 50 μM phosphorus (P) (+P). (a) Sampling of root segments of cluster roots at different developmental stages (×1); (b) a close-up of meristem initiation of cluster roots (×4); (c) a sketch of cluster-root sampling; (d) expression of xanthine dehydrogenase in root segments of cluster roots at different developmental stages. 1–5, zone 1–5; –PP, –P proteoid (cluster) root; –PN, –P normal root; –PL, –P leaf; +PP, +P proteoid (cluster) root; +PN, +P normal root; +PL, +P leaf.

cluster roots at different developmental stages from emergence to senescence (Figs 2, 3). A similar result was also obtained by quantification of endogenous NO using the ESR spectra (Fig. 5). SNP is the most commonly used NO donor in plants (Pagnussat *et al.*, 2003; Creus *et al.*, 2005; Velikova *et al.*, 2005), although SNP by-products might have some unwanted effects (Neill *et al.*, 2003). In the present study, addition of the NO donor SNP promoted P deficiency-induced lateral-root and cluster-root growth (Fig. 6). By contrast, the stimulating effects of NO on lateral-root and cluster-root growth were not observed under conditions of P sufficiency (data not shown), suggesting a complicated interaction between NO and P status in regulating lateral-root and cluster-root growth. These results suggest that P deficiency is an important factor in the control of cluster-root formation, and that NO may intensify the stimulating effect of P deficiency on cluster-root formation. The mechanisms underlying the interaction between NO and P deficiency in the control of cluster-root formation need to be further investigated. It has been demonstrated that auxin is an essential phytohormone that regulates cell division and elongation (Blilou *et al.*, 2005). There is evidence that auxin modulates P deficiency-induced cluster-root formation (Gilbert *et al.*, 2000; Neumann *et al.*, 2000; Skene & James, 2000). It has been reported that NO plays a key role in auxin-induced adventitious root development in cucumber (*Cucumis sativus*; Pagnussat *et al.*, 2003), and that NO can also increase root hair length and density in lettuce (*Lactuca sativa*) (Lombardo *et al.*, 2006). In a previous study, Zhao *et al.* (2007a,b) reported that high external nitrate concentration-induced inhibition of root growth in maize (*Zea mays*) is mediated by NO. Given that P deficiency is an important factor in cluster-root formation, and that root development and growth are closely related to NO, our results indicate that NO may be involved in P deficiency-induced cluster-root development.

Previous studies indicated that endogenous NO production in plants is mainly catalysed by the NOS-like protein and NR (Neill *et al.*, 2003; Wilson *et al.*, 2008). In the present study, it was found that the endogenous NO concentration in cluster roots at varying developmental stages was not affected by the NR inhibitor tungstate (Fig. 4). These results suggest that NR-mediated NO production may not be involved in the P deficiency-induced cluster-root formation. The results are in agreement with the previous report that nitrate uptake under P deficiency is strongly suppressed in *Lupinus albus* and other plant species

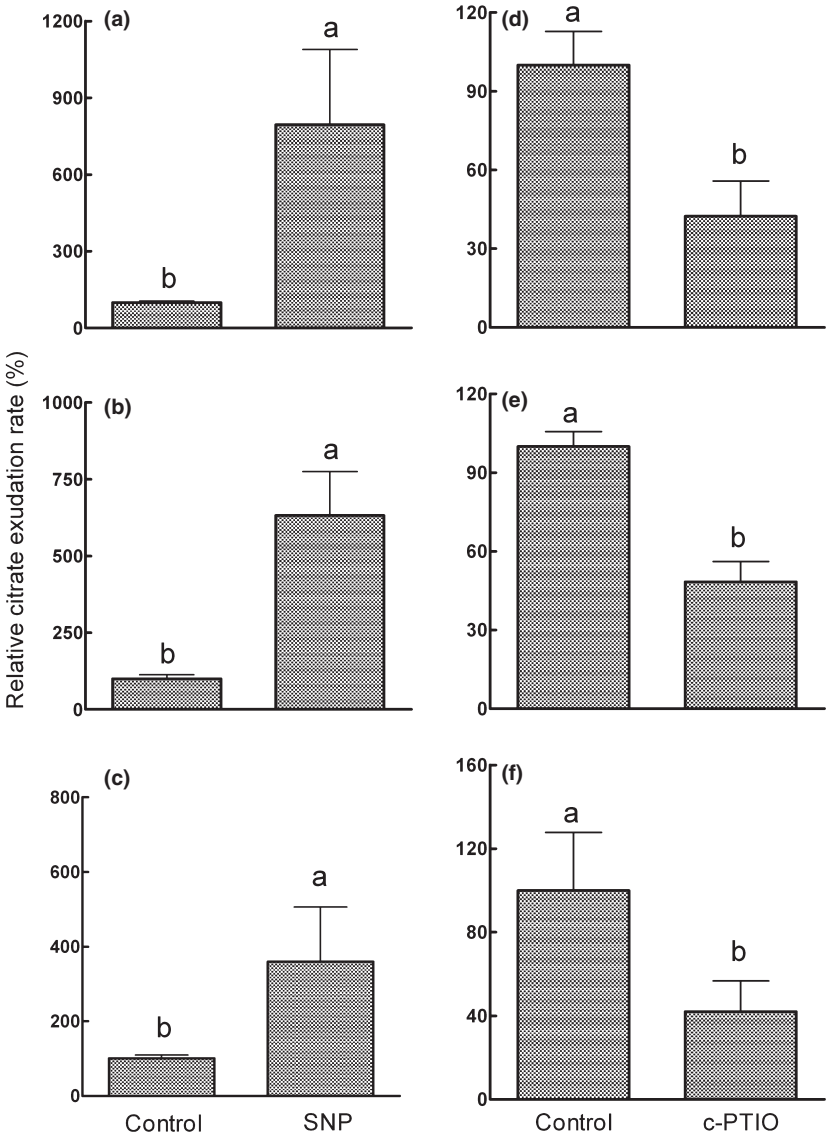

Fig. 8 Effect of the nitric oxide (NO) donor sodium nitroprusside (SNP) and the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) on citrate exudation from different root segments of phosphorus (P)-deficient white lupin (*Lupinus albus*). Roots of white lupin were incubated in 0 or 50 μM SNP solution for 24 h, or in 0 or 100 μM c-PTIO solution for 2 h. After the incubation, the root tips (0–10 mm) (a, d), juvenile cluster roots (b, e) and mature cluster roots (c, f) were excised and citrate exudation was collected. The percentage of citrate exudation rates in different treatments relative to control was calculated. Data are the mean ± SE of four replicates. Means with different letters are significantly different ($P < 0.05$) with regard to treatment.

(Neumann *et al.*, 1999). By contrast, the mammalian NOS inhibitor L-NNA reduced NO production in cluster roots (Fig. 4), suggesting that the NOS-like protein may contribute to overall NO production in P deficiency-induced cluster roots. In addition to the NOS-like protein, the XOR inhibitor allopurinol decreased the NO concentration in juvenile and mature cluster roots (Fig. 4). It was also found that P deficiency up-regulated the expression of XDH as a form of XOR, especially in mature cluster roots (Fig. 7). As XDH is an enzyme involved in purin catabolism, the strong expression of *LaXDH* in mature cluster roots is probably related to remobilization of organic P in cluster roots before senescence (Massonneau *et al.*, 2001). It has been demonstrated that XOR is involved in NO production in mammalian cells (Harrison, 2002). There is no direct experimental evidence in support of the involvement of XOR in NO production in plants (Neill *et al.*, 2003). Taken together, these results suggest that both the NOS-like protein and XOR may play roles in NO production during P deficiency-induced cluster-root formation in white lupin. Our findings also highlight the potential role of XOR in mediation of NO production, in addition to the NOS-like protein and NR, in plant cells.

P deficiency not only induces cluster root formation, but also stimulates citrate exudation to mobilize sparingly available P forms in soil, and exudation of citrate in response to P deficiency is well documented in cluster roots of white lupin (Keerthisinghe *et al.*, 1998; Watt & Evans, 1999a; Neumann & Martinoia, 2002; Shen *et al.*, 2004, 2005). Given that NO is involved in P deficiency-induced cluster root formation, the authors then further investigated the effects of NO on carboxylate exudation from cluster roots. Citrate exudation was predominantly detected in the present study. It was found that citrate exudation from cluster

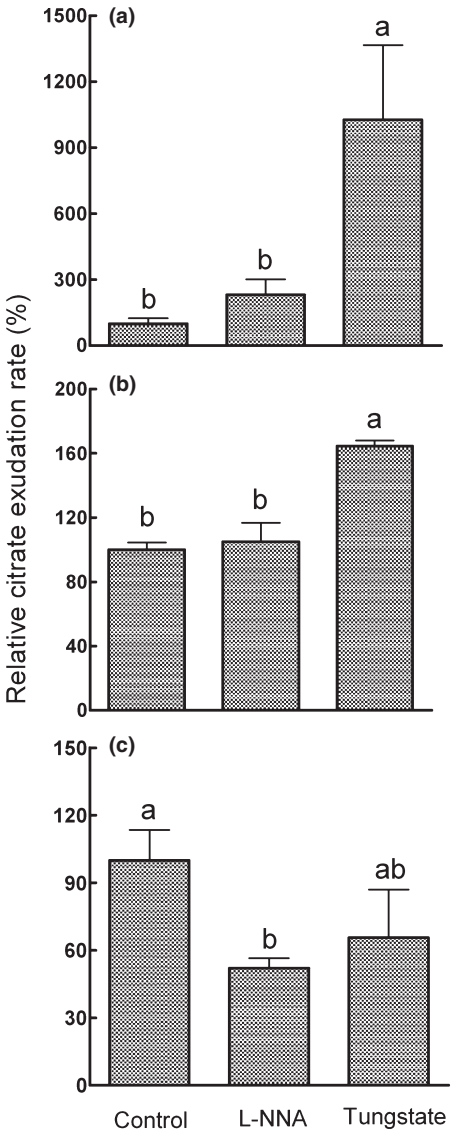

Fig. 9 Effect of the nitric oxide synthase (NOS)-like protein inhibitor L-NNA and the nitrate reductase (NR) inhibitor tungstate on citrate exudation from different root segments of phosphorus (P)-deficient white lupin (*Lupinus albus*). Roots of white lupin were incubated in 200 μM L-NNA or 100 μM tungstate solutions for 24 h. After the incubation, root tips (0–10 mm) (a), juvenile cluster roots (b) and mature cluster roots (c) were excised and citrate exudation was collected. The percentage of citrate exudation rates in different treatments relative to control was calculated. Data are the mean ± SE of four replicates. Means with different letters are significantly different ($P < 0.05$) with regard to treatment.

roots was closely associated with NO concentration, such that the NO donor SNP enhanced and the NO scavenger c-PTIO inhibited citrate exudation from cluster roots (Fig. 8). The increased citrate exudation from cluster roots of white lupin is consistent with an increased efflux of H^+, as a result of up-regulation of H^+-ATPases in the plasma membrane (Yan *et al.*, 2002), as well as increased efflux of other cations, including K^+, Na^+ and Mg^{2+} (Zhu *et al.*, 2005). It was previously shown that NO increased plasma membrane H^+-ATPase activity in reed (*Phragmites communis* Trin) plants (Zhao *et al.*, 2004). Therefore, it is possible that the enhanced citrate exudation induced by NO may result from its effect on stimulating H^+-ATPase activity. Citrate efflux from cluster roots of white lupin is likely to occur through anion channels permeable to citrate (Neumann *et al.*, 1999; Zhang *et al.*, 2004; Wang *et al.*, 2007). In animal systems, NO was reported to be a widespread regulator of both plasma membrane and endomembrane Ca^{2+} and K^+ channels (Clementi & Meldolesi, 1997; Shin *et al.*, 1997). In plants, there are several papers reporting that NO regulates K^+ and Ca^{2+} channels in plants (Casolo *et al.*, 2005; Sokolovski *et al.*, 2005). Whether the stimulation of citrate efflux by NO results from its direct effect on anion channels remains to be determined. In addition, the observed stimulatory effect of NO on citrate exudation may result from its effect on citrate metabolism, such that NO enhances accumulation of citrate in cells of cluster roots, facilitating citrate efflux from cluster roots. The released citrate, as one of the most effective carboxylates for mobilization of sparingly soluble P forms in soils, is not only determined by the activation of specific anion channels but also by a metabolic shift from intracellular accumulation mainly of malate in juvenile root clusters to almost exclusive citrate accumulation in mature cluster roots (Kania *et al.*, 2003). Further, the stimulating effect of tungstate on citrate exudation in juvenile root clusters (Fig. 9) may be accounted for by a shift from accumulation of intracellular malate to accumulation of citrate in the juvenile root clusters, as demonstrated by Kania *et al.* (2003). Further studies on the effects of NO on intracellular carboxylate profiles during cluster-root development are warranted.

In conclusion, in this study, differential patterns of NO production were demonstrated in roots of white lupin depending on root zone, developmental stage and P nutritional status. NO may be involved in P deficiency-induced cluster-root formation and citrate exudation. It was further revealed that P deficiency-induced NO production may result from up-regulation of the NOS-like protein and XOR. The physiological basis of these processes and the regulatory mechanisms involved in them warrant further investigation.

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